



INDONESIA INTERNATIONAL INSTITUTE
FOR LIFE SCIENCES

Improving The Quality of Life

Protocol Number : No.001.005

PROTOCOL

A Handbook on *in vitro* Gene Expression Study for Cosmetic Testing

Laboratory
Authors

: Skinovation Centre, Indonesia International Institute for Life Sciences
: apt. Pietradewi Hartrianti, Ph.D.
Richard Sutejo, S.TP., Ph.D.
Marsia Gustiananda, S.Si., Ph. D.
Felicia Edgina Susilo
Jesslyn
Michael Jonathan



Protocol : A Handbook on *in vitro* Gene Expression Study for Cosmetic Testing

Number of pages : 28

Authors : apt. Pietradewi Hartrianti, Ph.D
Richard Sutejo, Ph.D
Marsia Gustiananda, Ph.D
Felicia Edgina Susilo
Jesslyn
Michael Jonathan

Cover Design : Sohali

Published by : ©I3L Press

Year of publication : 2022

Dilarang mengumumkan, mendistribusikan, mengkomunikasikan, dan/atau menggandakan sebagian atau seluruh isi buku ini untuk tujuan komersial tanpa izin tertulis dari pihak Departemen Farmasi, I3L dan I3L press

Table of Contents

Introduction	3
Material and Methods	3
In vitro model	3
Test Compound (Groups of study or experimental design)	3
Method	3
Method Origin	3
Method Principle	3
Description of The Method	4
Results Interpretation and Analysis	22
References	23

Foreword

This protocol handbook was written for the study of *in vitro* gene expression analysis for the purpose of cosmetic testing. The authors initially developed the protocol during their thesis project, which analyzes the effect of a cosmetic product on sensitive skin by evaluating the gene expression related to skin barrier integrity, inflammation, and nerve sedation. The protocol handbook was developed with the hope that the written procedures are able to be applied in the *in vitro* cosmetic testing field.

Date : 13th May 2022

1. Introduction

In this current era, the growth of the cosmetic industry has been shown to increase remarkably. One of the main reasons is increased awareness of beauty and skin health. However, before the developed cosmetic product is launched to the market, it is essential to test the ingredients of the product itself. Several cosmetic products had been identified with several hazardous ingredients, such as allergens, endocrine disruptors, or carcinogens, which leads to the importance of conducting a cosmetic testing procedure. Aside from that, cosmetic testing also is a common practice conducted to evaluate the efficacy of the incorporated ingredients (Nadeeshani Dilhara Gamage et al., 2022). Several regulations also have been established to regulate the mass production of safe cosmetic products, such as the Europe Cosmetic Regulation N^o1223/2009, which provides a solid framework for the safety of cosmetic products. Thus, its purpose and the established regulations highlight the importance of conducting cosmetic testing procedures prior to the release of the products to the market (Barthe et al., 2021).

Cosmetic testing itself can be conducted through several means: *in vitro*, *in vivo*, and clinical studies. Although *in vivo* studies using mice or rabbits have been regarded as the “gold standard” for cosmetic testing for a very long time, *in vitro* studies have gained more popularity these days due to several reasons. First of all, the ethical issue concerning animal testing has caused regulatory frameworks, such as the European Union (2013) to ban animal testing. Not only that, more and more people are now aware of the ethical issue concerning animal testing and as a result, become more inclined to choose “cruelty-free” products. Second, *in vivo* testing are oftentimes more expensive and time-consuming, making *in vitro* testing to be a more economical option. Lastly, up to this date, there is still no hard evidence demonstrating that animal testing can truly predict the human response (British Toxicology Society, 2020). Meanwhile, clinical testing is usually done at the latest stage of cosmetic testing after *in vitro* or *in vivo* study to truly recapitulate the effectiveness and safety of the cosmetic product in human beings.

Among many different types of *in vitro* testing, gene expression analysis has been considered a preferred method for numerous studies investigating the effect of a cosmetic product or active ingredients in skin cell culture (Kim et al., 2018; An et al., 2017; Choi & Hwang, 2019). Gene expression analysis allows researchers to specifically measure the effect of the cosmetic on genes that are known to be attributed to certain skin conditions, such as inflammation and skin barrier. When various genes are investigated together, researchers can

not only obtain a general understanding of the effect of the cosmetic but also investigate the mechanisms of how these effects occur.

This handbook aims to provide a guideline on how to conduct an *in vitro* gene expression study for cosmetic testing purposes.

2. Material and Methods

2.1. In vitro model

- Model : HaCaT cell line (immortalized human keratinocytes cells).
- Culture medium : Dulbecco's Modified Eagle Medium (DMEM)
Containing:
 - 2 mM L-glutamine
 - Pyridoxine hydrochlorideSupplemented with:
 - 3.7 gr/L sodium bicarbonate
 - 1% Penicillin-Streptomycin (Pen-Strep)
 - 10% fetal bovine serum (FBS)
- Culture condition : 37°C, 5% CO₂.

2.2. Test Compound (Groups of study or experimental design)

- Product
- Base
- API
- Negative control

The test compound can be adjusted as needed.

2.3. Method

2.3.1. Method Origin

The effect of the moisturizer was analyzed through a gene expression analysis by observing the expression of the related genes following a cell cytotoxicity study. These analyses were conducted based on the methods described by Kim and colleagues in 2018, with the title of "Skin Protective Effect of Epigallocatechin Gallate", which was modified and suited for this study's objectives.

2.3.2. Method Principle

The *in vitro* gene expression study of the moisturizer follows several main steps, which are the keratinocytes cell culture, homogeneity test, cytotoxicity test of the moisturizer, and gene expression analysis.

In this handbook, the keratinocytes cell line is used to depict the skin reaction to the moisturizer samples treatment since this cell line made up the majority of the epidermal layer of the skin. The cell line used for the analysis is the spontaneously immortalized human keratinocytes called HaCaT. The maintenance of the HaCaT cells follows the common cell culture methods and conditions, including cell thawing, cell washing, cell seeding and passaging, and cell cryopreservation.

The homogeneity test is included in the study to ensure that the given samples are fully homogeneous in the cell treatment media. Since the homogenization of the moisturizer samples in the media is known to be quite challenging, the homogeneity test employs several methods that are commonly applied during the homogenization process, such as manual stirring, vortexing, sonicating, and employing the use of homogenizer.

Prior to the gene expression analysis, the proper concentration of the samples for further application is determined through the cytotoxicity test, specifically the MTS assay. The cytotoxicity test is used to determine the suitable concentration of the samples that will not cause cytotoxic effects on the cells. MTS assay is applied due to its practicality and time-efficiency in comparison to other cytotoxicity tests, such as MTT assay.

The gene expression analysis is conducted to determine the expression of the related genes after the cell treatment with the samples. The gene expression analysis consists of RNA extraction, cDNA synthesis, and qPCR processes. These procedures are recommended to be conducted in the molecular biology laboratory due to the suitability of the consumables and equipment with the conducted procedures. The qPCR study is conducted to measure the gene expression, in which the method measures the relative expression of the related genes upon cell treatment compared to control (untreated). The qPCR products are evaluated and assessed through a melting curve analysis and agarose gel electrophoresis to check the product specificity and size.

2.3.3. Description of The Method

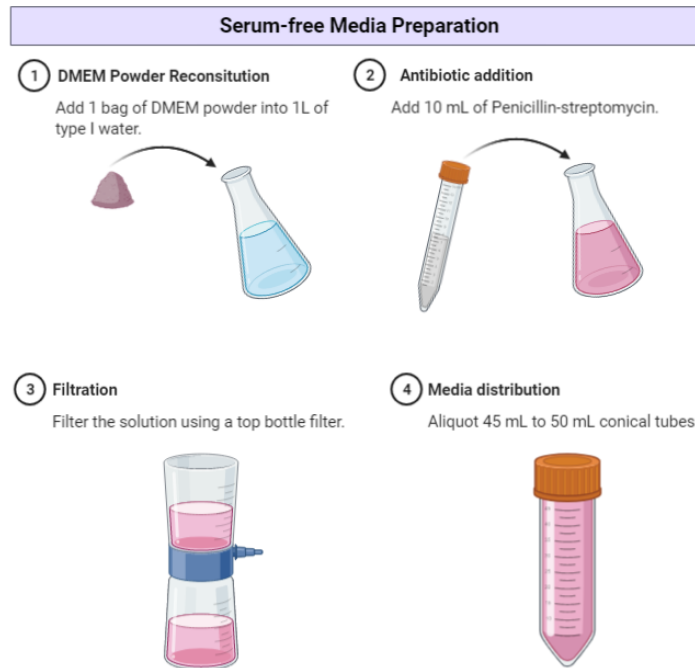
2.3.3.1. Biosafety Cabinet (BSC) Sterilization

All cell handling works should be performed inside a BSC.

1. Turn on the UV for at least 10 minutes.
2. Turn off the UV light and spray the BSC surface with 70% ethanol, then wipe with tissue paper.
3. Spray every item before placing it inside the BSC with 70% ethanol.
4. After the laboratory work is done, take out all items inside the BSC and spray the surface with 70% ethanol then wipe.
5. UV the inside of the BSC for at least 10 minutes.
6. Turn off the BSC.

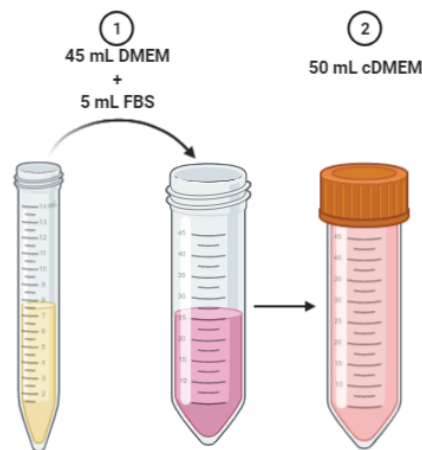
2.3.3.2. Cell Culture Media Preparation

2.3.3.2.1. Serum-free media (DMEM) preparation



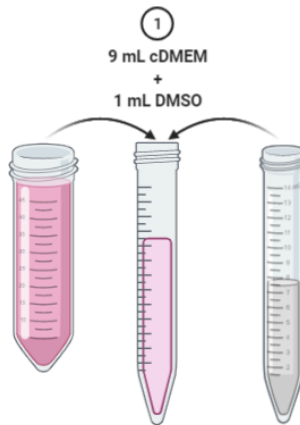
1. Dissolve one packet of DMEM powder in 1 L of Type I water.
2. Add 10 mL of Penicillin-Streptomycin to reach a final concentration of 1%, and mix well.
3. Inside a BSC, filter the DMEM + Pen-Strep using a top bottle filter.
4. If necessary, aliquot the DMEM + Pen-Strep into a 50 mL conical tube.

2.3.3.2.2. Complete media (cDMEM) preparation [10% FBS]



1. Mix the serum-free media with sterile fetal bovine serum (FBS) to reach a 10% final concentration. For example, to create 500 mL of cDMEM, mix 450 mL of serum-free media with 50 mL FBS, or to create 50 mL of cDMEM, mix 45 mL DMEM and 5 mL sterile FBS.
2. If necessary, aliquot the cDMEM into a 50 mL conical tube.

2.3.3.2.3. Cryopreservation media preparation [10% DMSO]



1. Mix cDMEM with sterile DMSO to reach a 10% final concentration. For example, to create 10 mL of cryopreservation media, mix 9 mL of cDMEM with 1 mL of DMSO.

2.3.3.3. Homogeneity Test

2.3.3.3.1. Moisturizer dose calculation

1. Convert the amount of moisturizer (product, base, and API) for skin application to the amount of moisturizer for treatment on a 24-well plate using the formula below:

$$\frac{\text{amount of cream on skin (g)}}{\text{amount of cream on 24-well plate}} = \frac{572 \text{ cm}^2 \text{ (face surface area)}}{1.9 \text{ cm}^2 \text{ (24-well plate surface area)}}$$

Note:

For the preliminary study, 1 g of product, which corresponds to 3.32 mg for 24-well plate, can be used as the foundation for determining the cell viability study concentration as this amount is generally recommended for topical cream usage (Kaewsanit et al., 2021).

2. Create a range of five different concentrations ranging above and below the amount of moisturizer (product, base, and API) based on the calculation at step a.
3. Repeat steps 1 and 2 as necessary to adjust the concentrations to be tested.

2.3.3.3.2. Homogenization process

It is recommended to conduct the homogeneity test with water first prior to the test in the cell culture media since the cell culture media is also water-based.

1. Weigh the desired amount of the samples in the conical tube according to the established recommended usage.
2. Add the necessary amount of water to the conical tube.
3. Mix the solution properly using a spatula.
4. Heat the solution in the 60-80°C water bath for approximately 20 seconds.
5. Vortex and homogenize the solution using vortex and homogenizer.
6. Repeat step 4-5 until the solution reached complete homogeneity.

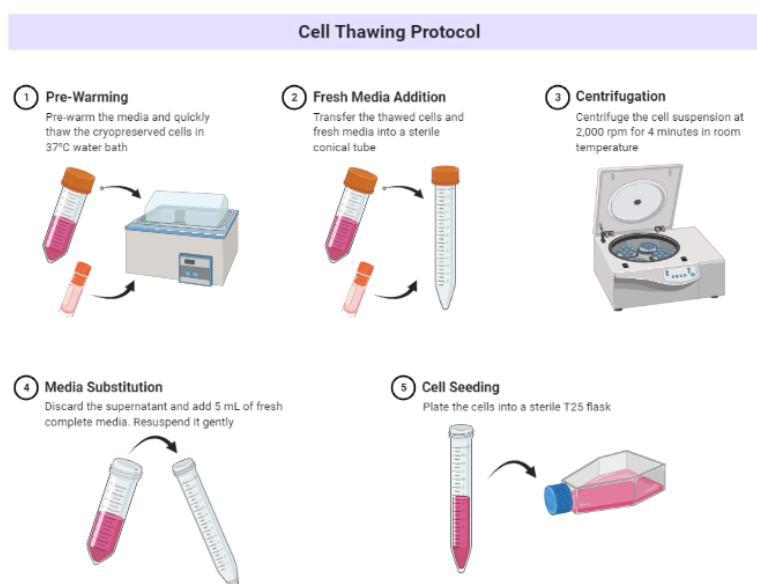
7. Let the solution stand according to the treatment time span to ensure no sedimentation occurs.
8. If complete homogeneity is unable to be achieved, increase the volume of the water.
9. If complete homogeneity is still unable to be achieved, incorporate the use of surfactant such as DMSO or Tween-80.

Note:

During the incorporation of surfactant, the surfactant should be mixed first with the samples prior to the addition of water or treatment media. Prior literature reading and the addition of surfactant group during cytotoxicity test are also needed to ensure the concentration of surfactant incorporated in the homogenization process is non-toxic for the cells.

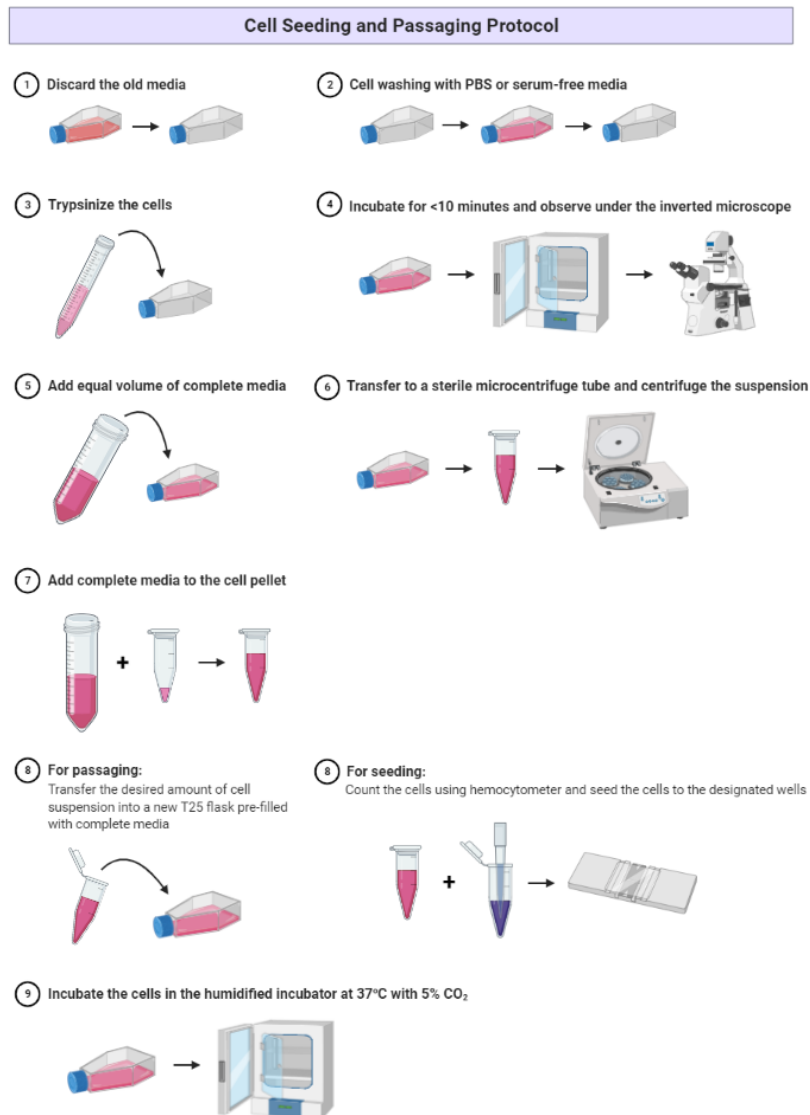
2.3.3.4. HaCaT Cell Maintenance

2.3.3.4.1. Cell Thawing



1. Pre-warm cDMEM in 37°C water bath.
2. Quickly thaw the frozen cells in 37°C water bath.
3. Aspirate the thawed cells (1 mL) and dispense them into a conical tube.
4. Add 9 mL of warm cDMEM to the cells while mixing gently.
5. Centrifuge the cells at 2000 rpm for 4 minutes at room temperature.
6. Discard the supernatant.
7. Add 5 mL of cDMEM, mix gently.
8. Plate the cell suspension to a T25 flask.

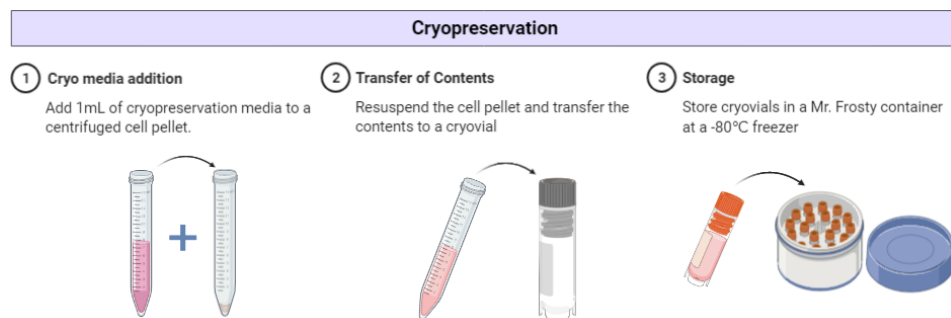
2.3.3.4.2. Cell Seeding and Passaging



1. Discard the old media from the T25 flask carefully without touching the cells layer into the 10% bleach solution.
2. Wash the cells using 2 mL PBS or serum-free media two times.
3. Add 1 mL trypsin into the flask to detach the cells.
4. Incubate the flask in the incubator for no more than 10 minutes. Check every 2 minutes to see if the cells have detached.
5. Observe the cells under the microscope to ensure proper detachment.
6. Add equal volumes of cDMEM (1 mL in this case) into the flask to inactivate the trypsin.
7. Resuspend the cell suspension gently and transfer the cell suspension into the 15 mL conical tube.
8. Centrifuge the cell suspension at 2000 rpm for 4 minutes.
9. Discard the supernatant gently without disturbing the cell pellet.

10. For cell seeding,
 - resuspend the cell pellet with 1 mL cDMEM or more (adjust according to the cell confluency observed; higher confluency will need a higher volume of cDMEM to ensure that the cells are easily countable).
 - Count the cells using a hemacytometer.
 - Mix the cell suspension with the required volume of cDMEM according to the desired density and seed in a well plate.
11. For cell passaging,
 - resuspend the cell pellet with 1 mL cDMEM.
 - Mix a fraction of the cell suspension (e.g., 1/2, 1/3, 1/4) with cDMEM to reach 5 mL final volume.
 - Transfer the cell suspension into the T25 flask.
12. Agitate the well plate/flask gently.
13. Label and incubate in a 5% CO₂ incubator at 37°C.

2.3.3.4.3. Cryopreservation

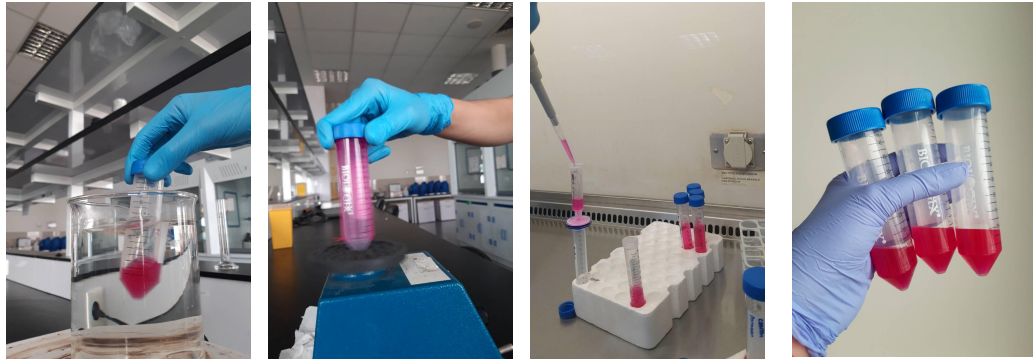


1. This protocol is intended to cryogenize one vial.
2. Do step 1 to 9 of 2.3.3.4.2.
3. Add cryopreservation media (consult 2.3.3.2.3. for cryopreservation media composition) to reach cell density of at least 1×10^6 cells. It is recommended to add 1 mL of cryopreservation media per cryovial.
4. Distribute the cell suspension equally into the cryovial.
5. Store the cells in Mr. Frosty in -80°C freezer.

2.3.3.5. Cell Viability Study

Treatment groups: product, base, and API (≥5 concentrations each), negative control, blank, surfactant (if needed).

2.3.3.5.1. Treatment Media Preparation



Heating of treatment media in a warm water bath

Homogenization of the treatment media with vortex

Sterilization of the treatment media by filtration process

The finished and homogenized treatment media

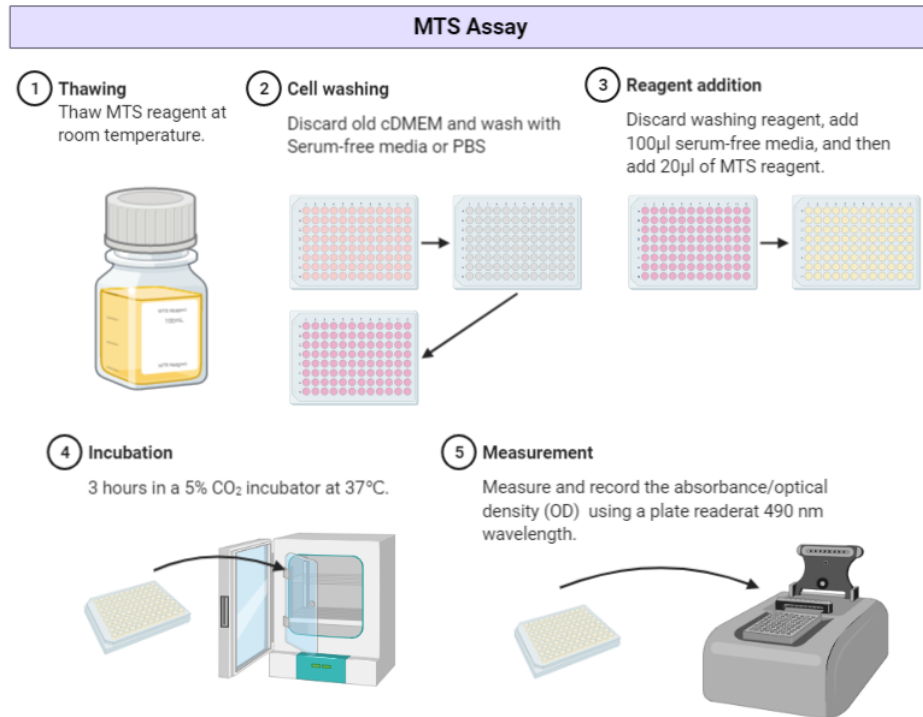
1. Place a beaker and a 50 mL falcon tube inside the beaker on the weighing scale
2. Weigh the corresponding amount of moisturizer (product, base, and API) for 10 mL of treatment medium into the falcon tube on the weighing scale.
3. Add 10 mL of serum-free media into the weighed moisturizer.
4. Heat the treatment media in a 60-80°C water bath for 20 seconds.
5. Vortex the treatment media for 10 seconds.
6. Repeat steps **d** and **e** until all of the samples were visibly dissolved.
7. Centrifuge the treatment media at 4,500 rpm for 30 minutes
8. Sonicate the treatment media for 6 hours.
9. Repeat steps **d** and **e**.
10. Filter all the treatment media using a 0.22 μm syringe filter to sterile 15 mL centrifuge tubes inside a BSC.
11. Store treatment media in 4°C if not directly used.

2.3.3.5.2. Cell Seeding and Treatment

1. Seed 10,000 cells per well of 96-well plate. Ensure that each treatment group (including negative control) has at least 3 replicates. Consult section **4.2** steps **a-j** for cell seeding protocol.
2. Prepare the highest concentration of the treatment media according to section **2.3.3.5.1**.
3. Serially dilute the highest concentration media to create the lower tested concentration of the samples.
4. Incubate cells at 5% CO₂ incubator at 37°C to reach ~80% confluency (for HaCaT cells, 24-48 hours is usually needed).
5. Remove the cell culture media and replace it with sterile treatment media.

- Incubate for 48 hours in a 5% CO₂ incubator at 37°C.

2.3.3.5.3. MTS Assay

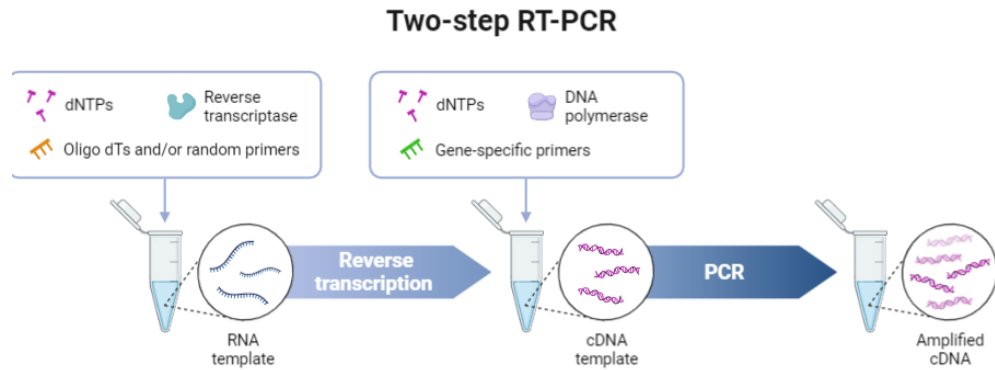


- Thaw the MTS assay reagent at room temperature.
- Discard the treatment media from the 96-well plate.
- Wash the cells gently by dispensing serum-free media to the wall of the well and then discarding it.
- Add the appropriate amount of serum-free media to each well depending on the kit used. For CellTiter 96® Aqueous One Solution Cell Proliferation Assay, add 100µl serum-free media to each of the wells.
- Add the MTS reagent.
- Add the same amount of serum-free media and MTS reagent to 3 empty wells to create blank wells.
- Incubate for 3 hours in a 5% CO₂ incubator at 37°C.
- Measure and record the absorbance/optical density (OD) at 490 nm wavelength using a plate reader.
- Calculate the cell viability with the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD sample} - \text{OD blank}}{\text{Mean OD control} - \text{OD blank}}$$

2.3.3.6. Gene Expression Analysis

Treatment groups: product, base, and API (1 concentration each), negative control



2.3.3.6.1. Treatment Media Preparation

1. Prepare the treatment media following section 2.3.3.5.1. Use the noncytotoxic concentrations of moisturizer (product, base, API) were chosen from the MTS assay. Consult the **result and interpretation section** to determine the noncytotoxic concentration of the moisturizer.

2.3.3.6.2. Cell Seeding and Treatment

1. Seed 50,000 cells per well of 24-well plate. Consult section 2.3.3.4.2. steps 1-10 for cell seeding protocol.
2. Incubate cells at 5% CO₂ incubator at 37°C to reach ~80% confluency (for HaCaT cells, 24-48 hours is usually needed).
3. Replace the old media with treatment media.
4. Incubate cells in a 5% CO₂ incubator at 37°C for 48 hours.

2.3.3.6.3. RNA Extraction

1. Follow the RNA extraction protocol according to the manufacturer's instructions. GENEzol™ TriRNA Pure Kit (GZX100) was used for this study.
2. Measure the RNA concentration and purity (A260/A280) with a Nanodrop spectrophotometer. Good RNA purity should be around 2.0.
3. Check the RNA integrity by conducting 1% agarose gel electrophoresis. Two bands which correspond to 28S and 18S rRNA should be present in intact RNA. Consult section 2.3.3.6.5.5. for the protocol to do agarose gel electrophoresis.
4. Store RNA samples at -80°C when not in use.

2.3.3.6.4. cDNA Synthesis

1. Follow the cDNA synthesis protocol according to the manufacturer's instructions. Thermo Scientific RevertAid First Strand cDNA synthesis Kit (#K1622) was used for this study. It is recommended to design the experiment so that each tube of the sample contains the

same amount of template RNA (e.g., 200 ng). This will ease the calculation for the following qPCR experiment.

2. Store the cDNA samples at -80°C when not in use.

2.3.3.6.5. qPCR

2.3.3.6.5.1. Primer Design

1. Open NCBI (<https://www.ncbi.nlm.nih.gov/>).
2. Click the drop-down menu on **all databases** and click **gene**.
3. Type in the name of the gene of interest.
4. Scroll down to the NCBI Reference Sequences (RefSeq) tab.
5. Locate the FASTA file of the mRNA of the gene of interest under the mRNA and protein subtab with the designator "NM_".
6. If there is more than one mRNA transcript, be sure to include all of the transcripts.
7. Open NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).
8. Select Primers common for a group of sequences.
9. Paste all of the transcripts of a gene of interest into the dialogue box.
10. Adjust the primers into the following settings:

Primer Parameters	Value
"PCR product size"	60-200 bp
"Primer size"	18-30 bp; 20bp optimum
"GC content"	40-60%
Primer sequence similarity with other genes	Not tolerable
"Maximum number of primer pairs to screen"	2000 pairs
"Maximum amount of GC at the 3' end of the primer"	2 bp
"Exon junction span"	Primer may span an exon-exon junction
"Intron inclusion"	Yes (can be adjusted)
"Max self complementarity at 3'"	1 bp
"Max pair complementarity at 3'"	1 bp
Primer location	Near 3' end

11. Ensure that the result of the Primer-BLAST is specific to the gene of interest by looking at the products on the intended targets section and making sure that the results only contain the gene of interest

- Ensure that the primer is predicted to anneal close to the 3'-end of the mRNA target sequence.

2.3.3.6.5.2. Primer Efficiency

- Dilute the synthesized cDNA into five concentrations (1X, 1/4X, 1/16X, 1/64X, 1/256X) using nuclease-free water. Each PCR reaction has a maximum of 50 ng of DNA, so 1X would be the 50 ng of cDNA.
- Conduct qPCR based on section **2.3.3.5.5.3**.
- Open Google Sheets or Microsoft Excel.
- Create a sheet and input the Ct values for each of the cDNA dilutions similar to the figure below.

Tube	Ct 1	Ct 2	Avg Ct	Sample Quantity	Log (sample quantity)
A	35.77	34.57	35.17	50.0000	1.70
B	32.9	33.37	33.135	12.5000	1.10
C	33.43	32.74	33.085	3.1250	0.49
D	32.51	33.92	33.215	0.7813	-0.11
E	33.87	28.82	31.345	0.1953	-0.71

- Plot the Average Ct values against the log₁₀ of the sample quantity.
- Calculate the slope of the graph using the "SLOPE" formula and input the average Ct values and the log₁₀ of sample quantity.
- Calculate the R-squared of the graph using the "RSQ" formula and input the average Ct values and the log₁₀ of sample quantity.
- Calculate the primer efficiency using the formula below:

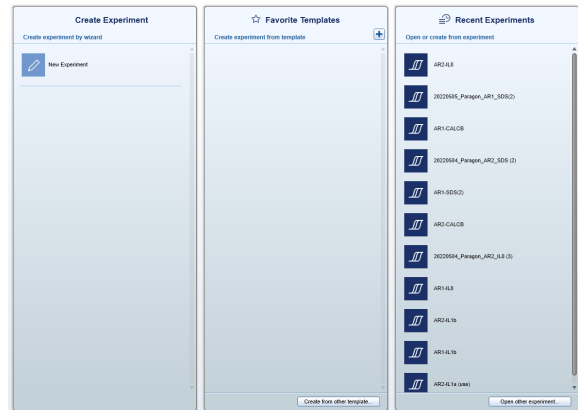
$$\text{Primer efficiency} = 10^{\left(-\frac{1}{\text{Slope}}\right)-1} \times 100$$

2.3.3.6.5.3. qPCR for Gene Expression Analysis

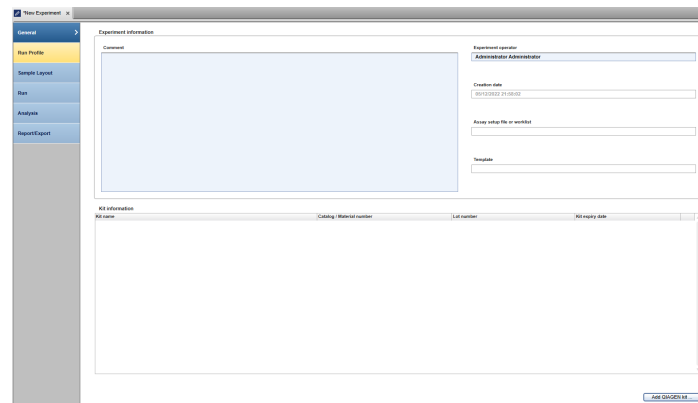
Follow the qPCR protocol according to the manufacturer's instructions. QuantiNova™ SYBR® Green PCR kit was used for this study.

- Clean the bench and the equipment with a bleach solution to eliminate exogenous DNA.
- Thaw the needed reagents, primers, and cDNA samples.
- Put all of the thawed reagents, primers, and cDNA samples in the ice bath.
- Make the master mix of the reagents and primers in sterile 1.5 mL microcentrifuge tubes.
- Transfer the master mix to each 0.2 mL PCR tube and add the corresponding cDNA samples.
- Vortex and spin down the PCR reaction mixtures prior to loading to the PCR machine.
- Load the samples into the PCR machine. In this study, the machine used for the gene expression analysis was the RT PCR Rotor-Gene Q.

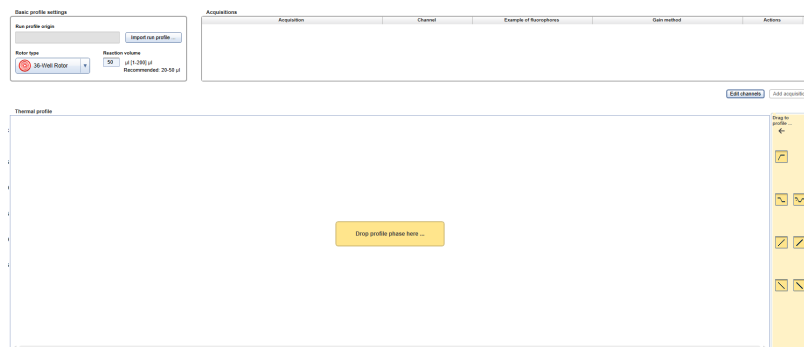
8. Open the Q-Rex software for Rotor-Gene Q.
9. Click “New Experiment”



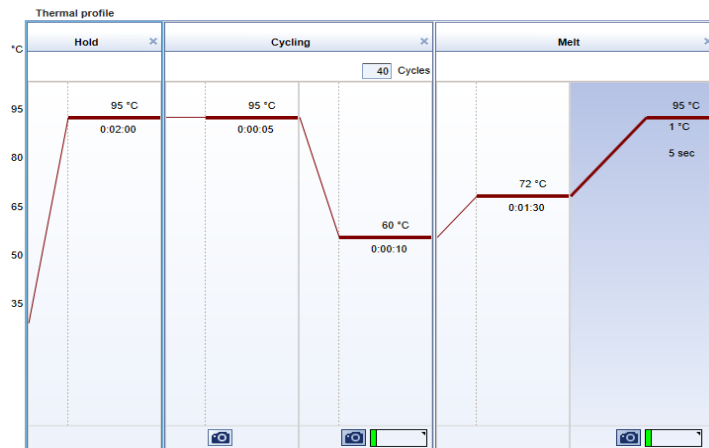
10. Click “Run Profile”



11. Drag and drop the phases needed for the PCR (Hold, two-steps cycling, and melt).



12. Change the reaction volume to 20 µL.
13. Set the thermal profile as the picture below



14. Click the sample layout tab and label the samples.

General					Green + Melt		
Tube	Color	Style	Name	Type	Target	Conc.	Unit
1	Red	---		Not in use	Green	0	None
2	Blue	---		Not in use	Green	0	None
3	Green	---		Not in use	Green	0	None
4	Purple	---		Not in use	Green	0	None
5	Orange	---		Not in use	Green	0	None
6	Black	---		Not in use	Green	0	None
7	Pink	---		Not in use	Green	0	None
8	Light Blue	---		Not in use	Green	0	None
9	Light Green	---		Not in use	Green	0	None
10	Cyan	---		Not in use	Green	0	None
11	Dark Blue	---		Not in use	Green	0	None
12	Yellow	---		Not in use	Green	0	None
13	Dark Green	---		Not in use	Green	0	None
14	Dark Purple	---		Not in use	Green	0	None
15	Black	---		Not in use	Green	0	None
16	Red	---		Not in use	Green	0	None
17	Blue	---		Not in use	Green	0	None
18	Green	---		Not in use	Green	0	None
19	Purple	---		Not in use	Green	0	None
20	Orange	---		Not in use	Green	0	None
21	Black	---		Not in use	Green	0	None
22	Pink	---		Not in use	Green	0	None
23	Light Blue	---		Not in use	Green	0	None
24	Light Green	---		Not in use	Green	0	None
25	Cyan	---		Not in use	Green	0	None
26	Dark Blue	---		Not in use	Green	0	None
27	Dark Green	---		Not in use	Green	0	None
28	Dark Purple	---		Not in use	Green	0	None
29	Black	---		Not in use	Green	0	None
30	Red	---		Not in use	Green	0	None

15. Start the run by clicking the start run button at the bottom-right corner of the screen.

16. Set the threshold according to the machine's "auto-threshold" setting.

17. Calculate the relative expression of the genes using the Pfaffl method by incorporating the primer efficiency test results.

$$\text{Relative gene expression} = \frac{(\text{Primer efficiency of GOI})^{\Delta Ct_{GOI}}}{(\text{Primer efficiency of HKG})^{\Delta Ct_{HKG}}}$$

GOI: gene of interest; HKG: housekeeping gene.

18. Store the PCR products at -80°C when not in use.

2.3.3.6.5.4. Quality Control

1. When creating the run profile for the qPCR reaction, add melting curve analysis to the end of the run.
2. After the qPCR is done, conduct 2% gel electrophoresis to confirm the correct PCR product size. Consult section **2.3.3.6.5.5.** for the protocol to do a 2% agarose gel electrophoresis.

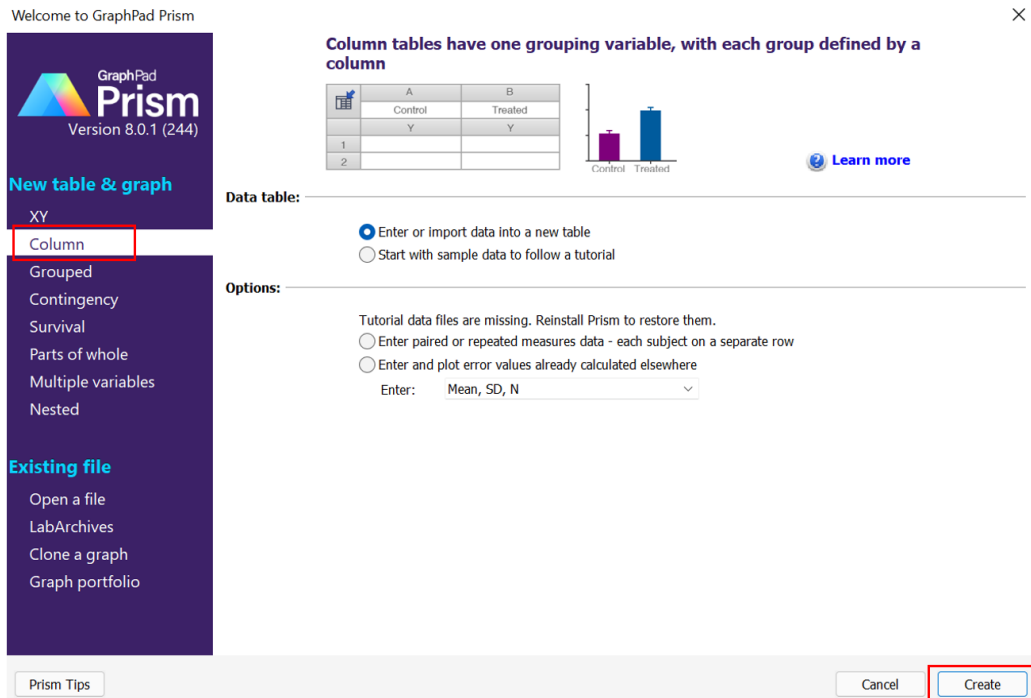
2.3.3.6.5.5. Agarose Gel Electrophoresis

1. Weigh 400 mg of agarose powder and add it to 40 mL of 1X TAE buffer in an Erlenmeyer flask for 1% agarose gel intended for RNA integrity check or 800 mg of agarose powder to the same 40 mL of 1X TAE buffer for 2% agarose gel electrophoresis intended for PCR product specificity check.
2. Microwave the mixture for 1 minute and mix halfway through and at the end by swirling the flask. Stop heating the mixture once the solution is clear.
3. Let the solution cool down for 5 minutes before adding 4 μ L of SYBR Safe DNA gel stain.
4. Swirl the flask to mix the gel stain.
5. Assemble an agarose gel casting tray.
6. Pour the solution into the mold.
7. Wait for 30 minutes.
8. Assemble a gel electrophoresis tank and power supply.
9. Pick up the gel with the casting tray and put it in the gel tank
10. Pour 1X TAE buffer solution into the tank until the maximum line.
11. Mix 5 μ L samples (RNA or DNA) with 1 μ L of loading dye before adding them to the wells of the agarose gel.
12. Run the gel at 100V; 400 mA; 45 minutes.
13. Visualize under a gel imager.

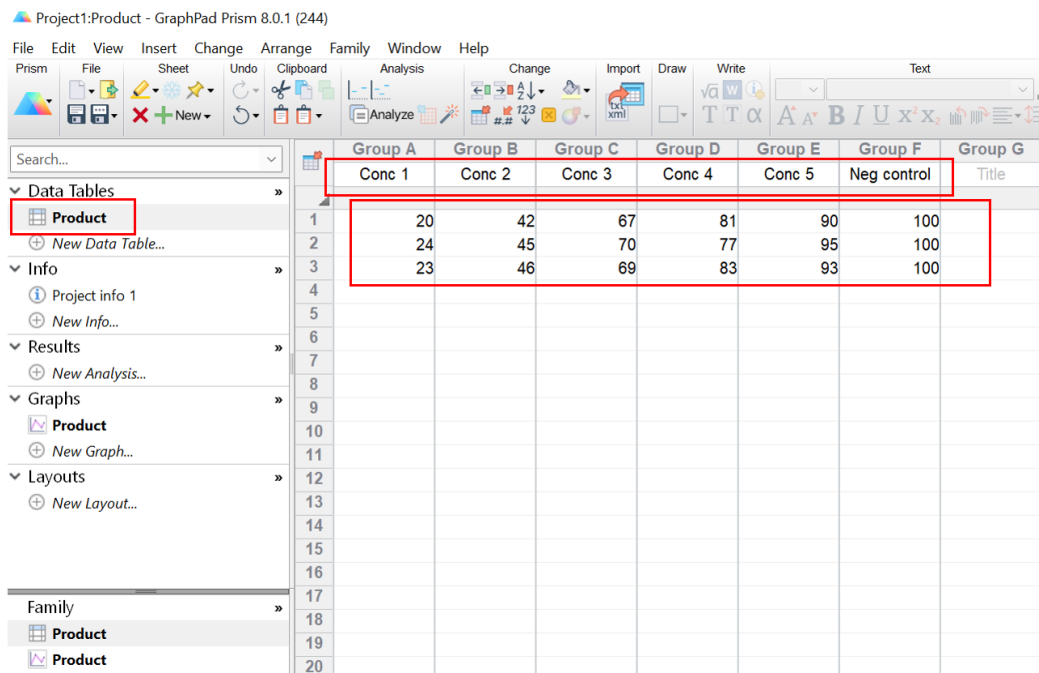
2.3.3.7. Statistical Analysis

Statistical analysis of the data can be done using any preferred or available statistical analysis software. This handbook will explain the steps required to conduct statistical analysis using GraphPad Prism 8.

1. Open GraphPad Prism 8 application
2. Under the “New table & graph” menu, choose “Column”.
3. Click “Create”.



4. Rename the label of the data label and group title.
5. Paste the dataset for each group.



- To test the distribution of each dataset, conduct Saphiro-Wilk test. This method is chosen due to its suitability for small sample size analysis (Mishra et al., 2019).
6. Click "Analyze".

Project1:Product - GraphPad Prism 8.0.1 (244)

File Edit View Insert Change Arrange Family Window Help

Prism File Sheet Undo Clipboard Analysis Change Import Draw Write Text

Search...

▼ Data Tables

- Product
- New Data Table...

▼ Info

- Project info 1
- New Info...

▼ Results

- New Analysis...

▼ Graphs

- Product
- New Graph...

▼ Layouts

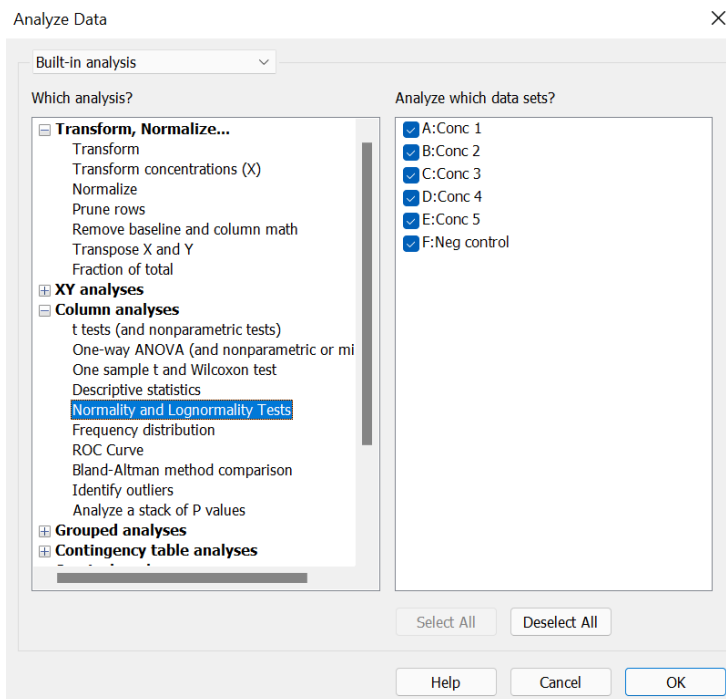
- New Layout...

Family

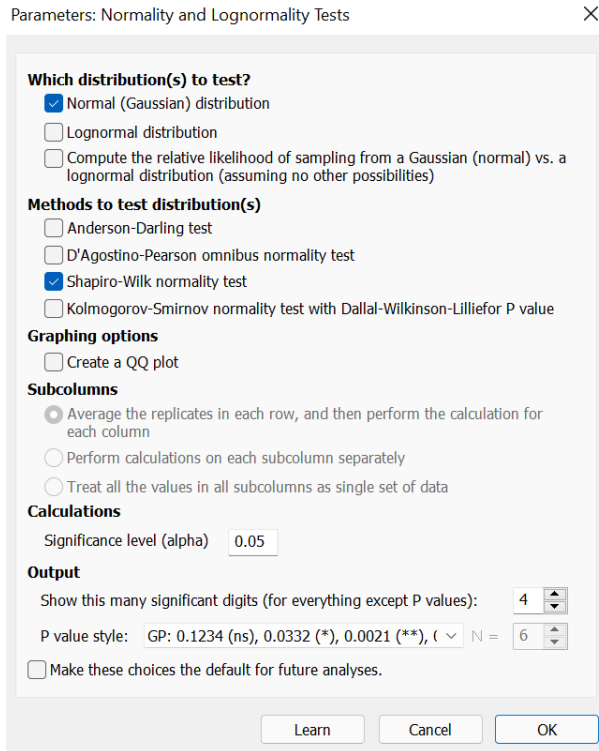
- Product
- Product

	Group A	Group B	Group C	Group D	Group E	Group F	Group G
	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Neg control	Title
1	20	42	67	81	90	100	
2	24	45	70	77	95	100	
3	23	46	69	83	93	100	
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							

7. Under the “Column analyses”, pick “Normality and Lognormality Tests”. Click OK.



8. Under the “Methods to test distribution(s)”, pick “Saphiro-Wilk normality test”. Click OK.



9. Check if all the dataset are normally distributed.

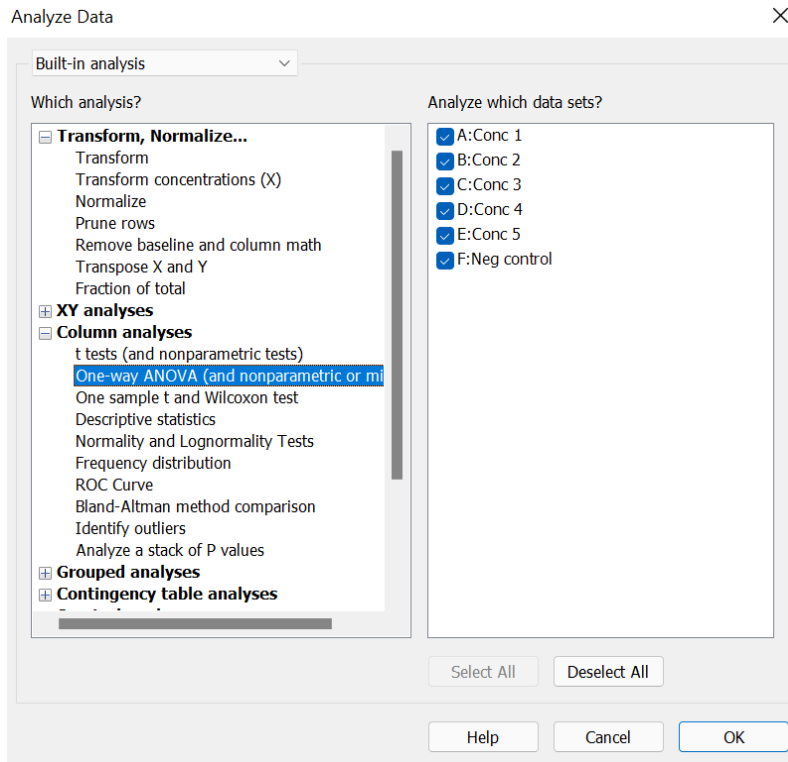
Project1:Normality and Lognormality Tests of Product - GraphPad Prism 8.0.1 (244)

	A	B	C	D	E	F
	Conc 1	Conc 2	Conc 3	Conc 4	Conc 5	Neg control
1	Test for normal distribution					
2	Shapiro-Wilk test					
3	W					
4	0.9231	0.9231	0.9643	0.9643	0.9868	0.8176
4	0.4633	0.4633	0.6369	0.6369	0.7804	0.1572
5	Passed normality test (alpha=0.05)? Yes					
6	P value summary					
6	ns	ns	ns	ns	ns	ns
7						
8	Number of values					
8	3	3	3	3	3	3
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

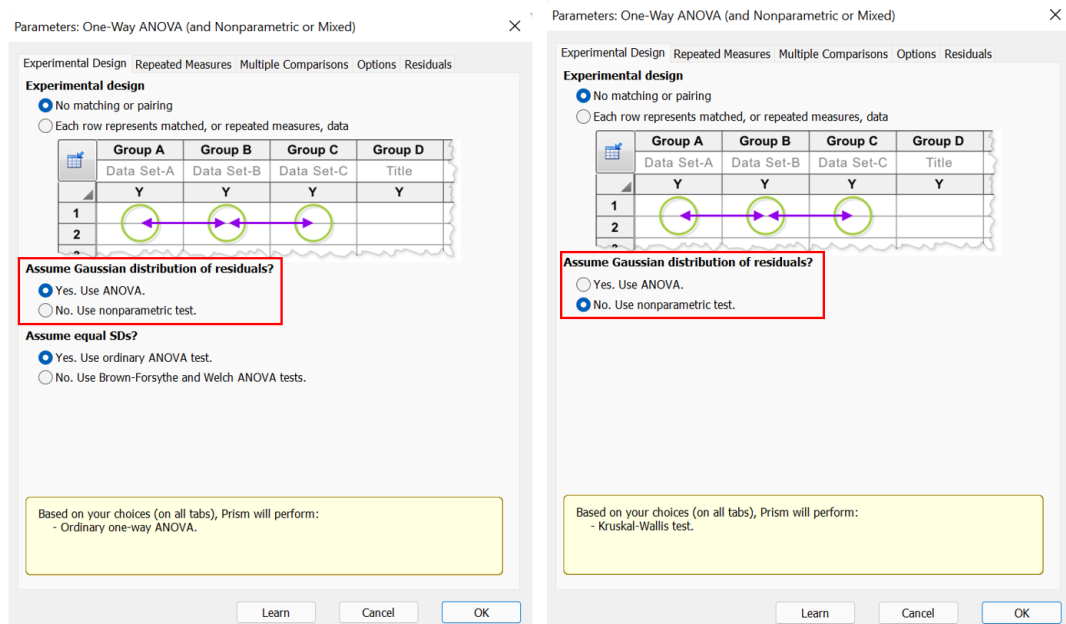
- If all dataset are normally distributed, conduct one-way ANOVA with Dunnett post hoc test. Otherwise, conduct non-parametric Kruskal-Wallis ANOVA with Dunn's post hoc test.

10. Return to the data table. Click Analyze.

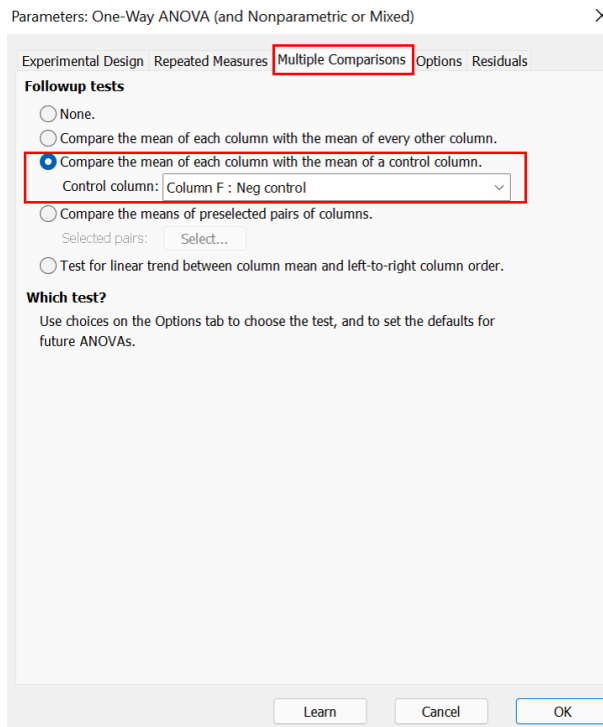
11. Under the "Column analyses", pick One-Way ANOVA (and nonparametric or mixed). Click OK.



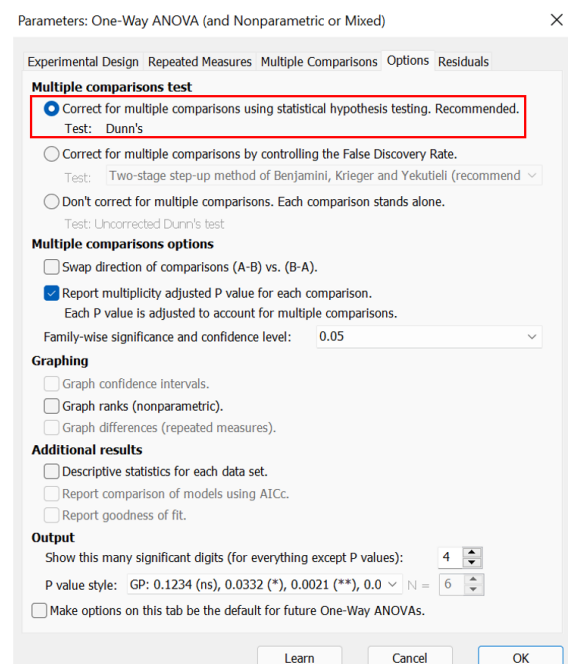
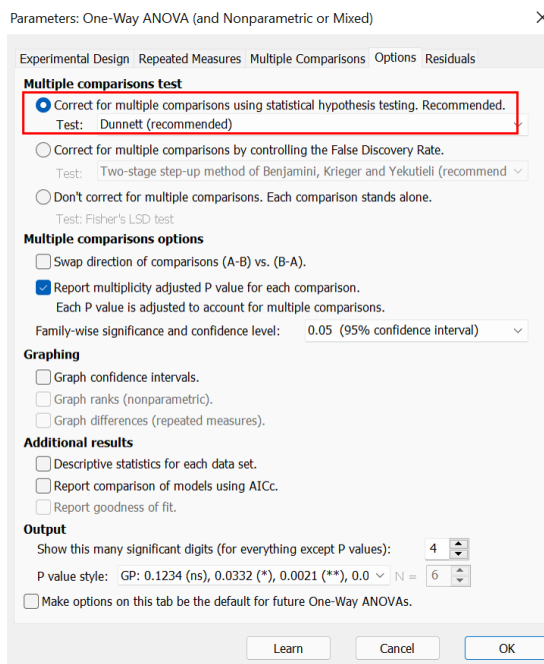
12. Depending on the normality test, pick “Yes. Use ANOVA” or “No. Use nonparametric test”.



13. Move to the “Multiple Comparisons” tab. Choose the “Compare the mean of each column with the mean of a control column.”, and choose the negative control as the control column.



14. Move to the “Options” tab. Check to ensure correct multiple comparisons test: Dunnett for One-way ANOVA, Dunn’s for nonparametric ANOVA. Click OK.



15. On the results page, move to the “Multiple comparisons” tab to see the result of each comparison. Pay attention to the “Significant?”, “Summary”, and “Adjusted P Value”.

Project1:Ordinary one-way ANOVA of Product - GraphPad Prism 8.0.1 (244)

File Edit View Insert Change Arrange Family Window Help

Prism File Sheet Undo Clipboard Analysis Interpret Change Draw Write Text Export Print Send LA Help

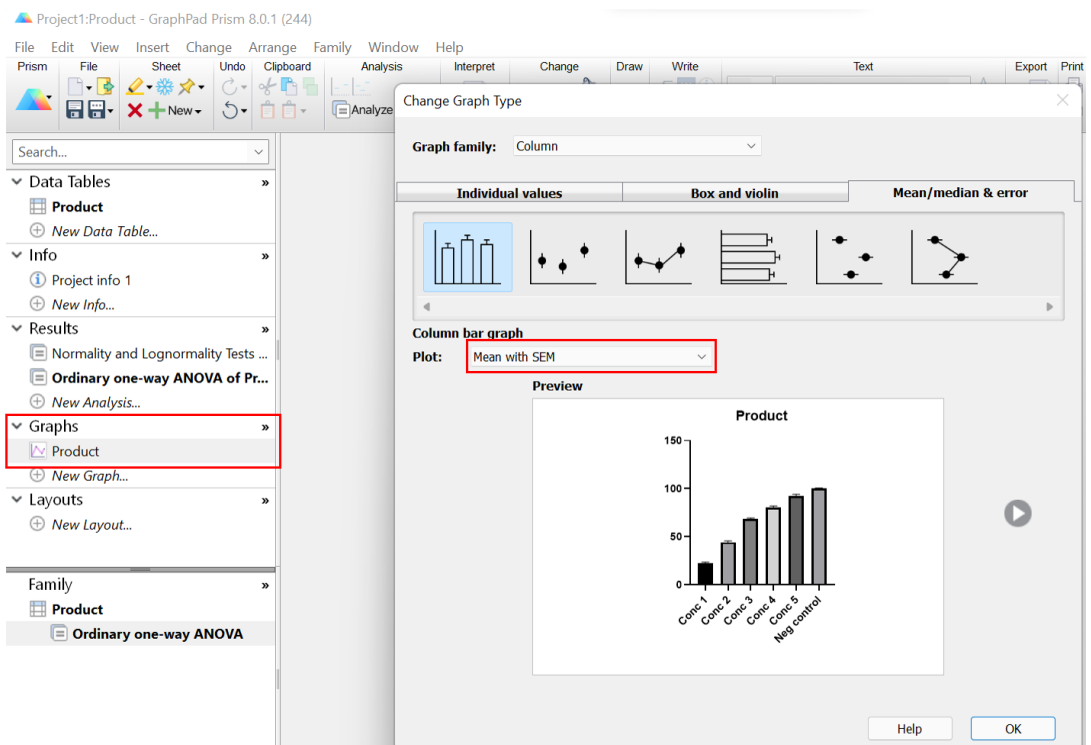
Search... ANOVA results Multiple comparisons

Ordinary one-way ANOVA
Multiple comparisons

1	Number of families	1							
2	Number of comparisons per family	5							
3	Alpha	0.05							
4									
5	Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary	Adjusted P Value	F-?			
6	Neg control vs. Conc 1	77.97	Yes	****	<0.0001	A	Conc 1		
7	Neg control vs. Conc 2	55.97	Yes	****	<0.0001	B	Conc 2		
8	Neg control vs. Conc 3	31.63	Yes	****	<0.0001	C	Conc 3		
9	Neg control vs. Conc 4	19.97	Yes	****	<0.0001	D	Conc 4		
10	Neg control vs. Conc 5	7.633	Yes	***	0.0009	E	Conc 5		
11									
12	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2		
13	Neg control vs. Conc 1	100.3	22.33	77.97	1.733	3	3		
14	Neg control vs. Conc 2	100.3	44.33	55.97	1.733	3	3		
15	Neg control vs. Conc 3	100.3	68.67	31.63	1.733	3	3		
16	Neg control vs. Conc 4	100.3	80.33	19.97	1.733	3	3		
17	Neg control vs. Conc 5	100.3	92.67	7.633	1.733	3	3		
18									
19									

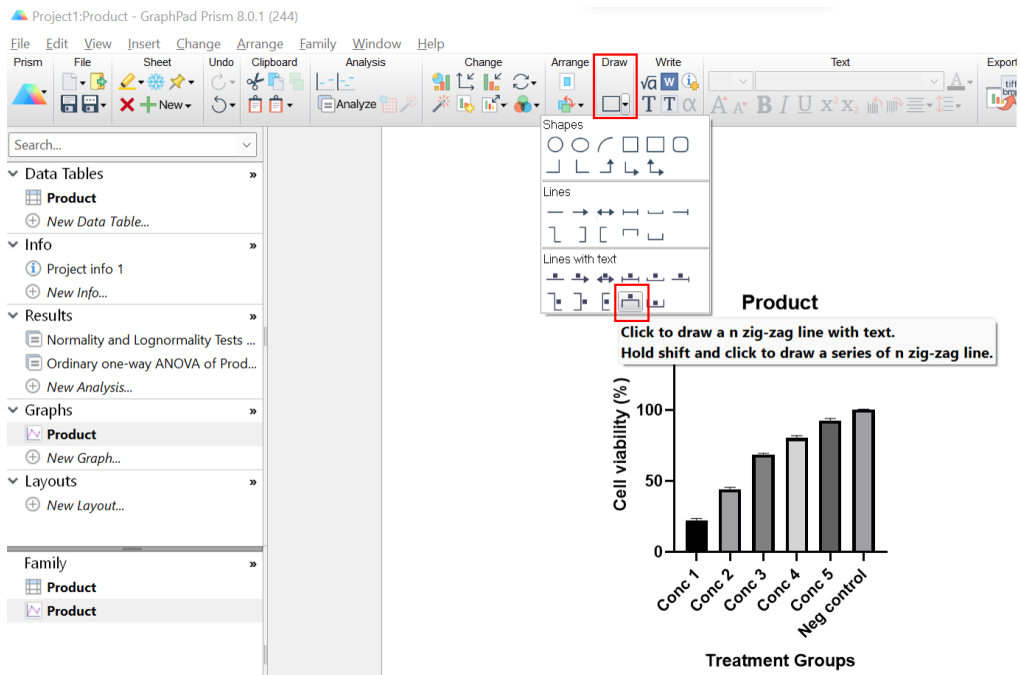
- Generate data comparison graph.

16. Click on the desired data group from the “Graphs” section. From the “Plot” dropdown menu, choose “Mean with SEM” or “Mean with SD”. Click OK.

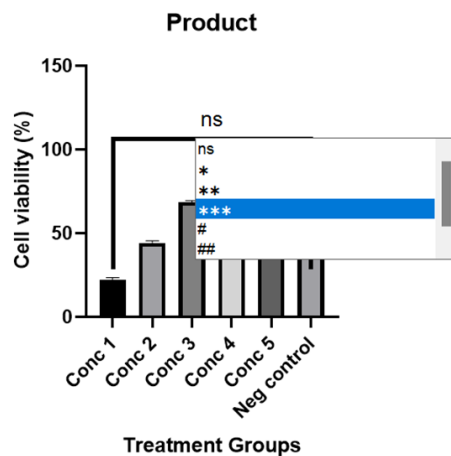


17. Change the title of the X and Y axis by clicking on the “X Title” and “Y Title”.

18. From the “Draw” dropdown menu, choose the line option as shown below.



19. Draw the line and choose the significant symbol based on the multiple comparisons result.



2.4. Results Interpretation and Analysis

2.4.1. Homogeneity Test

The aim of the homogeneity test is to ensure the complete homogenization of the samples in the treatment media. The samples' complete homogenization is essential for the complete pass-through of the samples through the syringe filter during the filtration process. Thus, the number of samples in the sterile treatment media will be similar to the unfiltered treatment media, which contributes to the generation of more reliable gene expression data. The complete homogenization of the samples is determined through visualization of the treatment media prior to the filtration process. The complete homogenization of the samples is concluded if the treatment solution is completely cloudy and no chunks of samples are visible in the treatment solution. The visible bit of samples denoted the incomplete homogenization of the samples in the media.

2.4.2. MTS Assay

The cytotoxic concentration of the treatment is determined based on ISO 10993-5:2009 and supported with a one-way ANOVA to confirm the statistical significance of the experiment. The final result of the MTS assay is a cell viability percentage which signifies the cytotoxicity effect of the moisturizer. The ISO 10993-5:2009 stated that a cell viability reduction of more than 30% is regarded as cytotoxic. Based on the tested range of concentrations, the highest concentration that was also within the range set by ISO 10993-5:2009 was chosen as the noncytotoxic concentration of the moisturizer.

2.4.3. Primer Efficiency

Because this handbook will utilize the Pfaffl (2001) method of relative gene expression calculation, primer efficiency analysis must be performed. This assay is done under the assumption that not all PCR reactions proceed with 100% efficiency. In other words, for every PCR cycle, the genetic material does not always double. The primer efficiency assay creates a standard curve of Ct values against the amount of cDNA in one PCR reaction. An R² value of ≈ 1 signifies a primer efficiency of $\approx 100\%$.

2.4.4. qPCR

The relative expression of the related genes is calculated using the Pfaffl method. The calculation is done by considering the normalization of the expression against the housekeeping gene and the efficiency score of the incorporated primers. Several statistical analyses are performed after the calculation of the relative expression, including the normality test and the determination of the significance of the results. The relative expression is compared between the treated and untreated cells to validate the presence of the effect after the cell is treated with the samples. The significant difference in the expression between the treated and untreated cells, which is determined by the p-value below 0.05, indicates that the samples indeed affect the expression of the tested genes. The significantly higher relative expression of the evaluated genes in the treated cells in comparison to the untreated cells denotes the elevated gene expression resulting from the treatment with the samples. On the other hand, the significantly lower relative gene expression in the cells that received treatment compared to the untreated cells indicates the reduction of the gene expression due to the given treatment of the samples to the cells.

3. References

- An, S., Cha, H. J., Ko, J. M., Han, H., Kim, S. Y., Kim, K. S., ... & Kim, S. Y. (2017). Kinetin improves barrier function of the skin by modulating keratinocyte differentiation markers. *Annals of Dermatology*, 29(1), 6-12.
- Barthe, M., Bavoux, C., Finot, F., Mouche, I., Cuceu-Petrenci, C., Forreryd, A., . . . Osman-Ponchet, H. (2021). Safety testing of Cosmetic Products: Overview of established methods and new approach methodologies (NAMS). *Cosmetics*, 8(2), 50. doi:10.3390/cosmetics8020050
- British Toxicology Society. (2020, May). *The Status of Alternative (in vitro) Approaches to Toxicity Testing*. <https://www.thebts.org/wp-content/uploads/2020/10/InVitro-Approaches-for-Toxicity-Testing-v1-May-2020.pdf>
- Choi, D. H., & Hwang, H. S. (2019). Anti-inflammation activity of brazilin in TNF- α induced human psoriasis dermatitis skin model. *Applied Biological Chemistry*, 62(1), 1-9.
- Holmes, A. M., Mackenzie, L., & Roberts, M. S. (2020). Disposition and measured toxicity of zinc oxide nanoparticles and zinc ions against keratinocytes in cell culture and viable human epidermis. *Nanotoxicology*, 14(2), 263-274. doi:10.1080/17435390.2019.1692382
- Kaewsanit, T., Chakkavittumrong, P., & Waranuch, N. (2021). Clinical Comparison of Topical 2.5% Benzoyl Peroxide plus 5% Niacinamide to 2.5% Benzoyl Peroxide Alone in the Treatment of Mild to Moderate Facial Acne Vulgaris. *J Clin Aesthet Dermatol*, 14(6), 35-41.
- Kim, E., Hwang, K., Lee, J., Han, S. Y., Kim, E. M., Park, J., & Cho, J. Y. (2018). Skin protective effect of epigallocatechin gallate. *International journal of molecular sciences*, 19(1), 173.
- Mishra, P., Pandey, C. M., Singh, U., Gupta, A., Sahu, C., & Keshri, A. (2019). Descriptive statistics and normality tests for statistical data. *Annals of cardiac anaesthesia*, 22(1), 67.
- Nadeeshani Dilhara Gamage, D. G., Dharmadasa, R. M., Chandana Abeysinghe, D., Saman Wijesekara, R. G., Prathapasinghe, G. A., & Someya, T. (2022). Global perspective of plant-based cosmetic industry and possible contribution of Sri Lanka to the development of Herbal Cosmetics. *Evidence-Based Complementary and Alternative Medicine*, 2022, 1-26. doi:10.1155/2022/9940548